5-9166

Report P-4965/FG-Final

CONTRACT REPORT NO. OSQ85-00179

BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

for

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May 22nd, 1986

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TABLE OF CONTENTS

		PAGE NO.
	EXECUTIVE SUMMARY	
1.	INTRODUCTION	1
2.	BACKGROUND	1
3.	OBJECTIVE	4
4.	STRATEGY	4
5.	METHODOLOGY	5
	a) Harvesting of T. ferrooxidans Cultures	5
	b) Preparation of Cell Suspensions for Predator Cultures	7
	c) Isolation of Predator Cultures	7
	d) Subculturing of Predator Cultures	8
	e) Culture Maintenance	8
	f) Culture Acclimation	8
	g) Sample Collection	9
6.	RESULTS AND DISCUSSION	10
	a) Selection of Sampling Sites	10
	b) Selection Criteria	11
	c) Isolation Tests for Predators	12
7.	CONCLUSIONS	14

APPENDIX I - TABLES

APPENDIX II - MICROSCOPIC EXAMAINTION OF TEST CULTURES

APPENDIX III - FIGURES

APPENDIX IV - BIBLIOGRAPHY

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BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

EXECUTIVE SUMMARY

The biologically catalysed generation of acid within tailings containing pyrite or other sulphide minerals is an insidious problem. Physical/chemical procedures to inhibit the growth of acid generating, autotrophic bacteria within a tailings pile are liable to disruption due to factors such as seismic activity, rainfall, erosion, etc. Biological control procedures to prevent the growth of acid generating organisms would not be as sensitive to environmental upsets and furthermore, would not be introducing a foreign chemical into the environment.

A previous study demonstrated that protozoa able to predate on Thiobacillus ferrooxidans could be isolated from tailings samples. However, the rate of predation was too low for an effective control procedure. This study has screened samples from selected sites including uranium tailings, high sulphur waste pulps, mill effluents and municipal sewage for more active protozoan predators towards T. ferrooxidans. However, more have been isolated with sufficient predatory activity to warrant further investigations. Based on these studies, it is proposed that the use of predatory protozoa to control the development of acid generating organisms in tailings may be difficult to implement. It is recommended that at the present time, the search for bacteriophage active against T. ferrooxidans be give precedence over a continuation of the search for protozoan predators.

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LE CONTROLE BIOLOGIQUE DES THIOBACILLUS FERROOXIDANS PAR DES ORGANISMES PREDATEURS

SOMMAIRE

La production d'acide catalysé biologiquement dans les résidus stériles contenant de la pyrite ou d'autres minéraux sulfurés est un problème complexe. Les procédés physiques et chimiques pour inhiber la croissance de bactéries autotrophes génératrices d'acide dans les résidus stériles sont assujettis à des facteurs tels que l'activité sismique, les pluies, l'érosion, etc. Par contre des procédés biologiques de contrôle pourraient être utilisés pour prévenir cette croissance d'organismes générateurs d'acide. Ces procédés ne sont pas aussi susceptibles de provoquer des bouleversements environnementaux. De plus, l'introduction d'un produit chimique étranger à l'environnement serait évitée.

Une étude antérieure a démontré que des protozoaires prédateurs de thiobacillus ferrooxidans peuvent être isolés d'échantillons de résidus stériles; cependant le taux peu élevé de prédateurs ne permet pas d'établir un procédé de contrôle efficace. Afin de trouver des protozoaires prédateurs de thiobacillus ferrooxidans plus actifs, on a examiné dans cette étude des échantillons prélevés dans des emplacements sélectionnés, comprenant des résidus stériles d'uranium, des minéraux sulfurés, des effluents d'usines ainsi que des eaux usées municipales. Suffisamment de prédateurs furent isolés pour justifier des recherches supplémentaires, bien que l'utilisation de protozoaires prédateurs pour contrôler le développement d'organismes générateurs d'acide dans les résidus stériles puisse sembler difficile à implanter.

Il est recommandé que présentement la recherche d'un bactériophage actif contre les thiobacillus ferrooxidans ait préséance sur la recherche dédiée aux protozoaires prédateurs.

BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

1. INTRODUCTION

This report is submitted in accordance with the terms and conditions of DSS Contract, Serial No. OSQ85-00179, File No. 09SQ23440-5-9166, whereby, the Ontario Research Foundation has undertaken a program to attempt to isolate predatory organisms with enhanced ability to prey on Thiobacillus ferrooxidans.

2. BACKGROUND

T. ferrooxidans has long been recognized as playing a large part in the generation of acid mine drainage. This autotrophic, iron-oxidizing bacterium, oxidizes ferrous iron and reduced sulfur compounds in pyritic minerals with the production of acidic ferric sulfate solution. This acidic solution helps to initiate the oxidative dissolution of sulfide materials, especially iron pyrite, in ore residues and tailings. As T. ferrooxidans continues the catalysis of the ferrous/ferric oxidation reaction in addition to the dissolution of the pyritic minerals, the acidity of the surrounding environment is maintained.

The severity of the environmental problem when the acidic effluents run into nearby streams, ponds and lakes, is often compounded as toxic metals are leached from the ores. Also, such metals may then be bioaccumulated into the food chain leading to potential health risks.

Many control technologies use physical or chemical methods to produce either an unfavourable environment for T. ferrooxidans or to insolubilize or to neutralize any potentially toxic elements. T. ferrooxidans is an obligative, aerobic microorganism (requiring oxygen) and requires a pH of 4.2 or less to support its active growth. Consequently, control techniques attempt to create anaerobic and/or alkaline environments with the tailings.

Some examples of these methods include capping, vegetation cover, addition of lime to make the environment highly alkaline and deepwater disposal. However, rainfall, erosion, earth tremors, etc., can result in physical damage to a barrier or cap or through leaching action, can lead to the gradual neutralization of the environment within the interstitial spaces of the tailings pile. This may result in restoration of conditions favourable for the growth of the acid producing organisms such as T. ferrooxidans. Climatic or geological factors reduce, therefore, the effectiveness of these methods for the long term stabilization of pyritic tailings.

Biological control methods offer the attractive feature of using biological agents with minimal environmental side effects since they act specifically against the thiobacilli, rather than modifying the tailings environment. It is desired that a controlled biological population can be established within a tailings pile such that it could be self generating as additions were made to the tailings pile, thus preventing the excessive development of acid-generating bacterial communities.

A successful biological control agent must be able to maintain an ecological environment within the tailings over an extended time period to prevent the establishment of a large population of acid-generating T. ferrooxidans. An ideal situation would be to identify and isolate as bacteriostatic agent, a predator for T. ferrooxidans. Most predators require a minimal concentration of "prey" for survival. Therefore, a "perfect predator" must not have a rate of predation that is too high. Otherwise, it will deplete the population of bacterial cells too rapidly, leading to its own demise from having eliminated its food supply.

The ideal situation is envisaged as to have a predator exist in a steady state with the T. ferrooxidans population, whereby the rate of predation would not exceed the bacteria's reproductive rate. Thus the microbial population would be maintained at a non-critical level so that little or not acid would be produced. Alternatively, the predator should be capable of encystment when bacterial numbers decrease and also readily excyst as the bacterial population begins to increase in number.

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Several factors will influence the effectiveness of this control technology. These include the minerology of the tailings, the ecology and interaction between bacteria and tailings particles, the population dynamics of the bacteria and the inoculated predatory organisms, the moisture balance and the specificity of the predator towards the target organism, T. ferrooxidans.

A previous program undertaken at the Ontario Research Foundation for Supply and Services Canada (DSS Contract File No. 26SQ.23440-3-9137) evaluated published data relating to the use of predatory organisms to control acid production in mining wastes, including protozoa, bacteriophage and possible bacterial predators such as Bdellovibrio sp. The data were inconclusive since none of the methods assessed was as yet proven technology. It had been noted by McCready (1984) that a flagellated protozoan rapidly reduced the numbers of T. ferrooxidans in a liquid culture by approximately 16 orders of magnitude within 16-24 hours at ambient temperature. Accordingly, a preliminary laboratory program was undertaken to attempt to isolate protozoa able to prey on T. ferrooxidans at pH values of 3.2 - 5.0.

Tests indicated that a flagellated protozoan isolated from tailings samples collected from the Nordic site at Elliott Lake, could produce a significant reduction in T. ferrooxidans populations in aqueous suspensions at pH values similar to those found in some mine wastes. However, the rate of predation was slow and the organisms did not reduce the bacterial numbers below approximately 10^6 cells/mL. This rate of predation would not be satisfactory for a control technology.

The goal of the current program has been to screen a wider range of sampling sites for the presence of more effective predators. Subsequently, candidate organisms would be compared for their efficacy to reduce the populations of T. ferrooxidans in liquid suspensions and attempts would be made to determine the effect of factors such as pH, sulfate and heavy metal concentration, size of prey population etc., on the adaptability and performance of such predators.

Sources of these predatory organisms would include mining wastes and tailings which are known potential acid sources but do not generate severe acid leachates. Other sources would be sewage treatment plants and other systems treating sulphur-containing wastes such as from pulp mills.

3. OBJECTIVE

The objective of this program has been to attempt to isolate more effective predatory organisms for T. ferrooxidans and subsequently to characterize the effect of pH, sulfate and heavy metal concentration, size of prey population, etc., on the survival and effectiveness of the candidate predators.

4. STRATEGY

Four phases were envisaged for this program with decision to proceed with any phase being dependent on the results of the earlier phases.

In Phase I of the program, samples would be collected from sampling sites selected in consultation with the Scientific Authority. Mine wastes and/or tailings locations would be identified as sources of predatory organisms. The criteria for such selection would include attempts to identify selected sites where expected acid effluents were not being encountered; sites treating effluents containing high concentrations of sulphur; and sites treating effluents with high organic carbon contents e.g. municipal and industrial wastes.

In Phase II, the samples collected would be screened to attempt to isolate potential predatory cultures using T. ferrooxidans as prey.

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Phase III would involve a comparison of the predatory efficiency of the organisms isolated in Phase II. The predatory activity of the isolated organisms towards T. ferrooxidans was to be compared in terms of percent kill versus unit time. Effects of various factors including pH, sulfate and heavy metal concentration, size of prey population, etc., on the survival and effectiveness of the predators was also to be investigated.

An overall assessment of the use of acidophilic heterotrophic bacteria, isolated from mine wastes, as prey and as a substrate for maintenance of the predator organism would be under taken in Phase IV. The ability to use such heterotrophic species to culture and maintain predators would eliminate the need to cultivate large volumes of T. ferrooxidans. The latter cultures do not afford a high yield of biomass and the recovery and clean-up of the cells is more complicated than for heterotrophic species.

The failure to isolate suitable predatory organisms in Phase II of the program led to this phase being extended to attempt to isolate a candidate organism. The eventual lack of success led to the decision to halt the program at this time.

5. METHODOLOGY

a) Harvesting of T. ferrooxidans Cultures.

A T. ferrooxidans suspension was required as prey to support the isolation and maintenance of predatory organisms. The cells were grown with gentle aeration in a medium containing dipotassium phosphate (0.4g), magnesium sulfate heptahydrate (0.4g), ammonium sulfate (0.4g) and ferrous sulfate heptahydrate (34.9g) in one litre of solution pH 2.20. Incubation was at room temperature (23^{o}C) with the cells being harvested as often as two times per week.

Three methods were used to recover and clean-up the T. ferrooxidans cells.

The initial procedure used centrifugation throughout. The Thiobacillus culture was centifuged at 3,800 r.p.m. for twenty (20) minutes at ambient temperature (Christ Model UJ3) to recover a cell pellet. The pellet was resuspended in sulphuric acid solution (pH 1.5) made up in sterile distilled water and spun at 2,500 r.p.m. at 4°C for five (5) minutes (IEC Model PR-2 Refrigerated Centrifuge). The supernatant suspension was decanted and retained. The orange-brown pellet was resuspended in sulphuric acid solution pH 1.5 and this suspension was again centrifuged for five (5) minutes at 2,500 r.p.m. The supernatant susupension was decanted and combined with the original supernatant. The pellet was discarded since it was composed almost entirely of insoluble iron compounds e.g. jarosite-type materials.

The combined suspensions containing the Thiobacillus cells were now centrifuged at 16,000 r.p.m. at 4°C for ten (10) minutes (IEC Model PR-2 Centrifuge). If any orange-red colour was detected in the pellet (i.e. iron compounds still present in the pellet), the pellet was resuspended in sulphuric acid solution pH 1.5 and the centrifugation procedure was repeated in its entirety. Otherwise, the pellet (beige in colour) were washed free of acid by suspending the cells in sterile distilled water and then recovering the cells by centrifugation. The final pellet was suspended in sterile distilled water and stored at 4°C until needed.

A second more rapid procedure for harvesting the Thiobacillus cells used vacuum filtration, with 0.45 micron sterile filters (47 mm diameter) to recover the cells initially from the culture suspension. The mixed cell/iron precipitates suspension was filtered and a small amount of sulphuric acid solution, pH 1.5 , was used to resuspend the cells and precipitates on the filter. From this point, the method for obtaining a suspension of T. ferrooxidans in sterile distilled water via centrifugation was the same as described in the first method.

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A final modification to the cell harvesting procedure was the use of high pressure filtration (Nucleopore 142mm Stainless Steel Filter Holder). This filter holder using 142mm Tuffryn^{R} filters (Gelman) could process 1.5 litres of culture suspension at one time. This expedited the initial harvesting of the T. ferrooxidans cultures. Cells and iron precipitates were washed from the filter(s) using the sulphuric acid solution pH 1.5 and the remainder of the purification method was as described in the previous method.

b) Preparation of Cell Suspensions for Predator Cultures

The harvested Thiobacillus cells were diluted with filter sterilized rural spring water to give a suspension with an absorbance of 0.55 (1 cm light path) at 600 mu. The pH of this suspension was adjusted as required with dilute sulphuric acid solution prior to addition to the tailings or sewage samples. For example, the Thiobacillus suspension was adjusted to pH 2.8 for addition to samples where an attempt was being made to recover potential predators effective at low pH values. If the sample had an original neutral or alkaline pH and it was uncertain that the protozoa would survive a drastic pH change, the Thiobacillus suspension was adjusted to pH 6.5.

c) Isolation of Predator Cultures

Potential protozoan samples (30mL) were transferred to large screw-capped test tubes and T. ferrooxidans suspension (10mL) adjusted to the desired pH value was added. The pH of the mixture was checked and adjusted if required by the addition of very dilute sulphuric acid solution. The screw cap was loosened to allow the exchange of gases. The samples were incubated at room temperature in the dark in order to minimize the risk of the growth of algal contaminants.

Incubated mixtures were examined every day or every second day. The decrease in the Thiobacillus population in a culture (if at all) was assessed by both visual and microscopic examination of the cultures. The microscopic examination used a phase contrast microscope at x100, x200 and x400 magnification. Wet mounts without coverslips were used so as to avoid affecting the mobility and viability of larger organisms.

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d) Subculturing of Predator Cultures.

Smaller zooflagellates were sought in this program. Several test cultures indicated the presence of active predator populations based on the decrease in the density of the bacterial suspension. However, in almost all instances where this depletion of bacterial cells was rapid (i.e. within three days) the reduction of number of T. ferrooxidans cells could be attributed to the presence of larger ciliated organisms.

Differential centrifugation was used to attempt to separate the larger ciliated organisms from the smaller zooflagellates and smaller ciliated species. Centrifugation at low speed was used (i.e. 2,500 r.p.m. for 3-5 minutes at $4^{o}C$; IEC Centrifuge). The centrifuged suspensions were sampled immediately with aliquots being withdrawn from close to the surface to reduce the risk of transferring the larger organisms which, due to their high motility, might be expected to rapidly redisperse in the total suspension. In some instances, it was necessary to repeat this procedure with successive cultures in order to eliminate the larger organisms.

e) Culture Maintenance

Cultures containing populations of smaller zooflagellates and ciliated protozoa were used to inoculate additional suspensions of T. ferrooxidans cells. Incubation conditions continued to be ambient temperature in the dark.

f) Culture Acclimation

Initial tests failed to isolate predators able to actively prey on T. ferrooxidans at pH 3.0 - 3.5 but in some instances rapid reduction of bacterial numbers was effected by cultures of smaller protozoa at pH 6.5 - 7.5. Attempts were made to acclimate these cultures to retain their predatory activity at lower pH values. To do so, successive cultures of a predator or mixture of predators were incubated with the pH value of the cell suspensions

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being decreased by 0.5 - 1.0 units per culture provided that positive predatory activity was observed at the previous pH value. The incubation period would depend on the individual culture but was generally significantly longer than that required with the original culture.

g) Sample Collection

Samples were collected at several sites including; Gulf and Shell Canada gas plants in the Pincher Creek - Waterton area of Alberta. Treated and untreated effluents were obtained of seepage and run-off from the sulphur obtained blocks and from drainage basins at pH 1.0 - 4.6.

Samples from waste treatment sites were collected at the Clarkson Waste Treatment Facility and the Oakville (East End) Waste Treatment Facility. Three types of sample were obtained at the Clarkson Plant namely, raw sewage, activated sludge and anaerobic sludge. Raw sewage was the only sample collected from the Oakville Plant. When relatively active protozoan cultures were isolated from the waste treatment facilities although none were found to be active at low pH values, a program was initiated whereby samples were collected from the Clarkson Plant twice weekly for a period of approximately six (6) weeks. The Oakville Plant was also sampled over this period but at less frequent intervals. This repetitive sampling was intended to allow for possible variations and fluctuations in the protozoan population in the sewage and sludges as the organic loadings vary with time.

Plant effluent samples were kindly supplied by a company producing corn starch and a poultry processing company.

6. RESULTS AND DISCUSSION

a) Selection of Sampling Sites

The sampling sites selected are shown in Tables 1-3. (Appendix I)

The Gulf and Shell plants in Alberta were selected since their processes for sulphur removal and storage lead to effluents containing significant concentrations of sulphur and reduced sulphur compounds, i.e., energy substrates for autotrophic acid-generating Thiobacilli. These sites encounter low pH acid drainage problems although the fact that the lowest value in the samples collected was 3.2, which suggested that some mechanism might be inhibiting the growth of the Thiobacilli.

In the Shell plant, all of the samples, with the exception of the Ross Creek sample (#8), were very acidic. This suggested that well established acid-producing biological cultures were present, i.e., predators if present were not able to control the generation of acid. Prior to inoculating test suspensions of T. ferrooxidans, the samples with pH values between 1.0 and 2.0 were adjusted to pH 3 with sodium hydroxide solution. In each case, a considerable amount of iron precipitates were formed as the pH was raised.

The E.B. Eddy Pulp Mill in Espanola uses Kraft pulping processes hence the effluents contain significant concentrations of reduced sulphur species. All of the samples were highly coloured (brown to deep brown) and the Wood Room Lagoon (sample #11) pH 4.2 had a strong odour of wood extractives. None of the samples had a distinct sulphur odour.

Samples collected at Inco plant in Sudbury included samples with no acid generation (pH 8.3 - 9.7) as well as samples with potential acid generating activity (2.7 - 3.3).

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The Nordic Site (Elliott Lake) was the source of the predator isolated in the previous study. The majority of the samples were from acid-producing sites (i.e., pH 2.7 - 3.2).

Two Waste Treatment Facilities were selected. The Oakville (East End) Plant and the Clarkson Plant treat predominantly a blend (50/50) of domestic and industrial sewage. The Oakville Plant treats a lower volume, than the Clarkson system.

When the above samples had failed to yield active predator cultures, two production facilities liable to have relatively high organic contents in their effluents were approached for samples. The operations were corn starch production and poultry processing. It was hoped to obtain samples from the treatment lagoons but unfortunately, the plant effluent samples were the only ones available. The investigation of high organic waste streams was introduced since it may be expected that bacterial populations in their waste treatment facilities will be relatively high. Such bacterial populations will in turn encourage the development of active predator populations, which was of interest for this study.

b. Selection Criteria

In a previous study, a predator culture was isolated from the Nordic Tailings (Elliott Lake) which could survive and actively predate on T. ferrooxidans at lower pH values (pH 2.5 - 3.5). However, the rate of predation was relatively slow and in liquid suspensions the bacterial population was only reduced by approximately 2 orders of magnitude, e.g., typically from approximately 10^8 to 10^6 cells/mL. The organism in this mixed predator culture, which were predominantly flagellated species, appeared to be unable to reduce the bacterial population below 10^5 - 10^6 cells/mL although they did appear to survive in this milieau.

c. Isolation Tests for Predators

For Phase II of the program, visual estimation of bacterial cell numbers in predator cultures were used throughout to assess the level of predatory activity. This procedure was satisfactory because the level of predation noted in most test cultures was generally poor or negligible. Where necessary, estimation of changes in cell populations was also based on microscopic examination. The original T. ferrooxidans suspension gave all cultures a decidedly opaque appearance at the outset of a test due to the numbers of bacterial cells present and the depth of the viewing field. In order to meet the strict criteria for possible predator cultures, the supernatant solution must be clear. A clear solution could still contain at least 10^3 T. ferrooxidans cells/mL.

The extensive list of tests conducted in this phase of the program and the general observations are shown in the tables included as Appendix II.

Predatory organisms were isolated in the program which could be maintained by subculturing into fresh T. ferrooxidans suspension at pH 6.5 - 8.0. Raw sewage samples obtained from both the Clarkson and Oakville Waste Treatment Facilities afforded very active predatory protozoan populations. Rapid depletion of the T. ferrooxidans population occurred in as little as three days with one sample of raw sewage from Clarkson. However, when transferred to T. ferrooxidans suspension at pH 3.5, there was a total loss of predator activity. Tests were initiated to attempt to acclimate the predatory protozoa in the cultures at pH 6.5 - 8.0 to survive at lower pH values. Stepwise reduction of 0.5 - 1.0 pH units for successive cultures was undertaken provided that predation was observed in a prior culture. A pH of 5.5 was the lowest value at which predation was observed. However, even at these values the rate of predation was very low. Below pH 5.5, predation was not detected and most protozoan cultures did not show any viability. Cultures maintained at pH 5.5 to determine if a predator species might acclimate and become dominant were not successful.

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Protozoan cultures isolated at pH 6.5 - 8.0 were initially mixed cultures of large and small ciliates together with flagellated species. Very active predation was always associated with the presence of the larger ciliated organisms but one concern was that the larger ciliated protozoa prevented the smaller flagellates (of greatest interest to the current program) from developing. Repetitive use of low speed centrifugation was required to separate the larger and smaller protozoa. Initially, no large protozoa would be seen in a culture following centifugation but after 24 - 48 hours' incubation with T. ferrooxidans suspension, the larger ciliated species would be re-established in the culture. Repetitive centrifugation of these cultures successfully separated the larger ciliated protozoa from the small ciliates and flagellates. However, the smaller organisms survival and dominance in a culture was accompanied by loss of the active predatory activity towards T. ferrooxidans. This loss of predation occurred following centifugation at natural pH values (pH 6.5 - 8.0) as well as lower pH cultures. Thus, while the smaller protozoa did survive, they did not significantly reduce the T. ferrooxidans population over periods of up to fourteen days.

The environmental samples collected from high sulphur wastes, mine wastes and/or tailings and pulp mills, yielded in some instances protozoa cultures able to survive at lower pH ranges (approx. 3.0 - 3.5). However, the rate of predation was always negligible and did not improve as additional subcultures were maintained at these pH values. Active predator cultures were obtained from some of these samples with pH values of 6.5 - 8.0. However, as in the case of the sewage protozoa, attempts to decrease the pH in a stepwise fashion led to the loss of all predation as well as the loss of all protozoan activity.

The samples of starch processing and chicken processing wastewater did not yield predatory cultures following centifugation to remove the larger ciliated protozoa. This lack of predation occurred at the natural pH values (6.0 - 7.5) of the samples. Such cultures consisted of very active populations of smaller ciliated protozoa and flagellates, which did not, however, appear to have significant ability to prey on T. ferrooxidans.

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Photographs of typical organisms in the mixed cultures of smaller ciliates and flagellates are shown in Figures 1 - 4 (Appendix III). These photographs were obtained by scanning electron microscopy (SEM) of culture samples which had been collected on Nucleopore filter membranes. The membranes + specimens were then fixed in glutaraldehyde, post fixed in osmium titroxide and critical point dried in carbon dioxide prior to SEM examination. The results with the SEM were significantly better than with the transmission electron microscope (TEM) which failed to yield examples of well defined smaller protozoa.

Figures 1 and 2 show a mixture culture of protozoa, several of which are flagellated, resting on a bed of bacterial cells consisting of T. ferrooxidans and bacteria present in the original inoculum (poultry process effluent). The protozoa in both photographs range from approximately 10 - 15 uM in length. Figure 3 and 4 show more highly magnified photographs of two flagellated organisms on a background of bacterial cells. In Figure 3, the biflagellated organism is approximately 12 uM long whereas the flagellate in Figure 4 is smaller being only approximately 6 uM in length. In both photographs, the short "stubby" cells are typical T. ferrooxidans while the longer rod-shaped bacteria originate from the inoculum.

7. CONCLUSIONS

In this study, the goal has been to isolate small protozoa able to prey on T. ferrooxidans at a much higher rate of predation than was obtained with an isolate in a previous study. Thus in laboratory cultures, organisms were sought which would reduce the numbers in a T. ferrooxidans suspension at pH 3.4 by 4-5 orders of magnitude within a few days. None of the mixed protozoan cultures isolated from a selected range of samples has met these specifications. It is therefore not feasible to proceed to the next phase of the program.

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Active predation at the level desired has always been obtained with mixed cultures of ciliated and flagellated protozoa at the pH range of 6.5 - 8.0. However, in most instances this very active predatory activity was attributed to the presence of larger (e.g., greater than 50 uM long) ciliated protozoa. Elimination of these larger species from the culture caused a substantial decrease in the rate of predation. The smaller flagellates and ciliates did predate at pH 6.5 - 8.0 but obviously the number of cells required per organism per day is too low to lead to a rapid decrease in the number of T. ferrooxidans cells in a suspension. Subculturing of these isolates failed to yield more active predatory species at the pH range of 6.5 - 8.0.

Gradual reduction of the pH of the T. ferrooxidans suspensions showed that the low level of predation is maintained by such cultures down to a pH of 5.5 but the rate continues to be too low to be practical and maintenance of cultures at this lower pH value failed to yield more active, acclimated species.

In some of the acidic water samples from the Gulf and Shell locations, or Elliott Lake, Nordic Site, smaller protozoa were identified and subcultured. However, as in the case of the higher pH isolates, the rates of predation were very low so as to be barely detectable after 2 - 3 weeks of incubation, i.e., reduction of T. ferrooxidans numbers by not more than 1 - 2 orders of magnitude within this period.

McCready (1984) has noted that small flagellated protozoa can show rapid rates of predation on liquid cultures of T. ferrooxidans cells at pH 2.2. In this study, such activity has not been noted. Samples were obtained from sites where the pH of all effluents might be expected to be acidic due to the activity of sulphur oxidizing bacteria. Some of these effluents/water samples had pH values in the 6.5 - 8.5 range which might be attributed to protozoa or some other factor causing the prevention of the development of active acid-producing bacterial populations in an otherwise

favourable environment. Such samples failed to yield protozoa with the high rates of predation that were desired. The protozoa in the more acidic samples represented organisms whose rates of predation were not high enough to cause the inhibition of acid-generating bacterial cultures. In essence the bacteria and protozoa co-exist in an acidic environment with the latter's predatory activity not exceeding the rate of growth of the bacteria, i.e., an ecological balance is maintained at pH 2 - 3.5.

A more extensive screening program might isolate a highly active predator species. McCready's protozoan contaminant in his culture originated from a contaminated distilled water system, i.e., not a source that one might have predicted. Thus, other "unpredictable" samples may be the source of the active predators. This was the logic for extending the sampling program of the waste treatment sites. Protozoan populations will fluctuate at the sampling sites due to factors such as organic loadings, bacterial numbers, volume, rate of aeration, etc. However, none of the extensive range of samples collected from those sites showed active predation of the desired magnitude. It is interesting to note that although samples were collected at regular intervals over several months, it was not until late winter that smaller ciliates and flagellates were isolated that would survive at pH 6.5 while being predatory towards T. ferrooxidans. Thus some changes appear to have occurred in the protozoan populations which typically will show cyclical patterns of growth and decline.

Based on the results of both this study and the previous study, it is difficult to invisage a control strategy for tailings piles based on these protozoa. The highly active protozoa species such as McCready encountered rapidly encysted when the T. ferrooxidans population declined below a certain number. He was, however, not able to induce excystment by replinishing with fresh T. ferrooxidans cells. To produce cells to maintain and cultivate such active predators would be very uneconomical and if a minimum T. ferrooxidans population is required along with some unknown factors to initiate excystment, significant acid generation would also be occurring, i.e., it would be a difficult balance to maintain.

ORI

Obviously, protozoa exist at the acid pH ranges of a mine environment. However, they are dependent on the presence of the T. ferrooxidans or other Thiobacilli as prey and hence would appear to live in a steady state relationship with the acid-producing bacteria, i.e., they do not control or prevent the acid generation. Moreover, their rates of predation are too low to justify seeking ways to increase substantially the size of these protozoa populations in the tailings environment. A further difficulty might be the development of active populations of larger ciliated species in response to the abundance of smaller protozoan which in addition to bacteria can serve as prey for the larger species. This appeared to occur in the small scale test with tailings samples in the previous study.

It was hypothesized that a desirable predator would be one that can co-exist with low levels of T. ferrooxidans cells (such that acid generation is minimal) but which can increase readily in numbers as T. ferrooxidans population increases. Such species could be maintained active in a tailings environment by the addition of supplemental bacterial prey other than T. ferrooxidans or other acid-generating species. Based on the results to date, the probability of this being attained is low. When one considers the additional factors such as the effect of particle size on the access of predator species to interstitial spaces within tailings piles, the effects of dehydration, access of moisture and air to maintain the predators etc., the failure to isolate predator species which are active in the controlled laboratory environment is seen to make such control technologies very improbable.

ORF

It is not deemed feasible to proceed with the program at the present time but it is recommended that the concept of biological control of acid-generating organisms be continued to be researched. This study has considered only one type of predator. The apparent need for a very extensive screening program to find "the organism" suggests that rather than directing such a program at the identification of a protozoan predator, the effort may best be directed at the search for alternative biological control agents for T. ferrooxidans such as bacteriophage.

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APPENDIX I

TABLES

Table 1. Sampling Sites in Western Canada

1. Gulf, Dingier Creek, off sulphur pad - soil, east
2. Gulf Process Water, Pond 6, pH 6, pH 7.5
3-1 Gulf Process Pond 8, Drainage, pH 3.2
3-2 Gulf Process Pond 8, pH 3.2
4-1 Gulf Process Pond 9, pH 7.0
4-2 Gulf Process Pond 9, pH 7.0
5-1 Shell Pincher Basin No. 1, pH 2.0
5-2 Shell "South of Sulphur Pile Basin No. 1, pH 2.0
6. Shell, Pincher Creek, Basin No. 2, pH 4.6
7. Shell, Pincher Creek, Sulphur Pile, run-off, pH 1.0
8. Ross Creek, Oct. 3/85, at Golf Course, pH 7.5

Table 2. Sampling Sites in Northern Ontario

9. E.B. Eddy, (Espanola), Main Pump Station, pH 7.1
10. E.B. Eddy, (Espanola), Treated Plant Effluent, pH 7.1
11. E.B. Eddy, (Espanola), Wood Room Lagoon, pH 4.2
12. Inco (Sudbury), Levack Tailings Main Pond, pH 9.7
13. Inco (Sudbury), C.D. area, pH 3.3
14. Inco (Sudbury), Acid Water, Dammed Creek, pH 2.7
15. Inco (Sudbury), Fresh Tailings, pH 8.3
16. Nordic (Elliott Lake), West Arm, North Swamp, pH 3.1
17. Nordic (Elliott Lake), West Tip, N. Nordic Lake, pH. 8.4
18. Nordic (Elliott Lake), East End, West Arm, pH 3.2
18a. Nordic (Elliott Lake), East End, West Arm sludge,
19. Nordic (Elliott Lake), Nordic Main Seepage Creek, pH 3.3
20. Nordic (Elliott Lake), Creek on Nordic Main, pH 2.7

Table 3. Sampling Sites in Southern Ontario

21. Clarkson Waste Treatment Facility (Mississauga) - Raw Sewage
22. Clarkson Waste Treatment Facility (Mississauga) - Activated Sludge
23. Clarkson Waste Treatment Facility (Mississauga) - Anaerobic Sludge
24. Oakville (East End) Waste Treatment Facility - Raw Sewage
25. Corn Starch Processing Effluent
26. Poultry Processing Plant Effluent

A P P E N D I X II

MICROSCOPIC EXAMINATION OF TEST CULTURES

MICROSCOPIC EXAMINATION OF TEST CULTURES

Nov 5/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS DAY
#9 E.B. Eddy, Main Pump Station pH 7.1	3.2	No protozoa to be seen
#10 E.B. Eddy, Treated Plant Effluent, pH 7.1	3.1	No motile protozoa
#11 E.B. Eddy,Woodroom Lagoon, pH 4.2	3.2	Mixed rods and Thiobacillus ferrooxidans. Possibly some yeast cells but no budding observed. No predators.
#12 Inco, Levack tailings, Main Pond, pH 9.7	3.2	Nothing to be seen except T. ferrooxidans (T.f.)cells
#13 Inco, C.D. Area pH 3.3	3.2	Nothing to see except T.f. cells
#14 Inco, Acid Water, dammed creek, pH 2.7	3.2	Nothing to see except T.f. cells
#15 Inco, fresh tailings, pH 8.3	3.2	Nothing to see except T.f. cells
#16 Nordic, West Arm, North (Swamp) pH 3.1	3.2	Some protozoa-generally circular and 8 to 10 times length of bacterial cell. Protozoa not motile.
#17 Nordic, West tip, North Nordic Lake pH 8.4	3.2	
#18 Nordic, East End, West Arm, pH 3.2	3.2	
#19 Nordic, Main Seepage Creek pH 3.3	3.3	
#20 Nordic, Creek on Nordic main, pH 2.1	2.1	Active protozoa seen
#18A Nordic, East End West Arm Sludge	3.2	

(cont'd)

Microscopic Examination of Test Cultures

Nov 12/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#1 Gulf, Dingier Creek off sulphur pad-soil	2.6	Mixed bacterial population, no identifiable protozoa but population does look reduced. Lots of particulate matter.
#1 Gulf, Dingier Creek off sulphur pad-soil	3.2	Mixed bacterial population, no identifiable protozoa to be seen, even at 1000X T.f. does look reduced.
#2 Gulf Process Water Pond 6, pH 7.5	2.9	No protozoa. High T.f. population.
#2 Gulf Process Water Pond 6, pH 7.5	7.0	Active motile protozoa - mixed population. Flagellated protozoa moved with torpedo-like motion, i.e. rotating on horizontal axis.
#3 Gulf Process Water Pond 8, pH 3.2	3.2	Very active ciliate population in high numbers. Size approx. 1/10th of field at 430 times magnification. Small flagellates seen but not present in large numbers, pear shaped cells.
#4 Gulf Process Water Pond 9, pH 7.0	3.2	No protozoa to be seen. High T.f. population.
#4 Gulf Process Water Pond 9, pH 7.0	6.5	Very clear. No T.f. left. Protozoan culture now defunct or encysted.
#5 Shell, Pincher Basin #1, pH 2.0	2.0	Reddish orange ring at air/liquid interface. (T.f. growth?)
#5 Shell, Pincher Basin South of Sulphur Pile pH 2.0	2.7	No reddish ring. Active culture of protozoa, mixed motile and non-motile. Motile protozoa very active.
#6 Shell, Pincher Creek Basin #2, pH 4.6	4.2	Mostly ciliates. No flagellates seen.
#7 Shell, Pincher Creek Sulphur Pile, Runoff pH 1.0	2.0	No active motile protozoa. Protozoa present did not show motility, i.e. probably very small ciliates.

(cont'd)

Microscopic Examination of Test Cultures

Nov 12/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#7 Shell, Pincher Creek Sulphur Pile, Runoff pH 1.0	2.8	Nothing to be seen. Iron precipitated out upon adjusting of the pH.
#8 Ross Creek, Oct 3 at Golf Course, pH 7.5	3.0	No active protozoa seen. T.f. still present.
#8 Ross Creek, Oct 3 at Golf Course, pH 7.5	7.5	Very clear. No T.f. left. Protozoa now defunct or encysted.

ORF

(cont'd)

Microscopic Examination of Test Cultures

Nov 13/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#3	3.2	Nothing. Cells tending to clump. No indication of predators. (OD 0.505)
#4	6.5	Active, motile protozoa present. ~1/10th field at 430X magn. moving linearly but rotate on horizontal axis. Look like ciliates. (OD 0.370)
#5	2.2	Mixed culture of motile and non-motile protozoa. Motile include biflagellates and smaller ciliates. (OD 0.432)
#6	4.6	No motile predators or cells clumping badly.
#5	3.0	Similar to #5 pH 2.2 but no motile cells to be seen. (OD 0.810)
#8	7.6	Suspension almost cleared. High population of highly motile flagellates, etc. with other protozoa. All small; no large ciliates (OD 0.125)

(cont'd)

Microscopic Examination of Test Cultures

From Nov 29/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#3	3.2	High population of T.f. No predators.
#4	6.5	Predators seen were all ciliates. No flagellates. Low population of T.f. Mixed bacterial population about 2X the size of T.f. cells.
#8	7.6	Low population of T.f. Ciliates were the only protozoa seen.
#5	3.0	High population of T.f. No predators seen.
#6	4.6	High population of T.f. No predators seen.
#5	2.2	High population of T.f. A few flagellates seen.
#16		Low population of T.f. Only saw 2 long "sticklike" flagellates.
#5	2.0	High population of T.f. A few flagellates seen.
#4	6.5	Low population of T.f. Many bipolar flagellates seen.
#8	7.5	High population of T.f. A few very large ciliates seen.
#14-2	2.5	High population of T.f. A lot of fast small predators - They appeared to be flagellates.
#3A-2	2.5	High population of T.f. Again a lot of small fast predators (Flagellates).
#14	2.8	Low T.f. population. Small fast predators seen.

ORI

(cont'd)

Microscopic Examination of Test Cultures

From Nov 29/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#21	2.5	Low T.f. numbers. No predators. A lot of debris present.
#19-2	2.6	Low T.f. population but no predators.
#15	3.0	Few T.f. cells. Iron particles observed. No protozoa.
#19	3.1	Low T.f. population. Some flagellates. Also some ciliates.
#20	2.9	Low T.f. population. Small flagellates seen.
#10	3.0	High T.f. population. No predators seen.
#9	3.0	High population of T.f. cells. No predators observed.
#11		High T.f. population. A few long thin flagellates. Slow motility.
#13	3.1	Low T.f. numbers. Large ciliates but no other predators seen.
#17	3.0	High population of T.f. No protozoa.
#18	3.0	High population of T.f. No protozoa.
#12		High population of T.f. No protozoa.
#1	2.6	High population of T.f. cells. Long narrow flagellates visible in association with the tailings.
#1	3.5	High population of T.f. cells. Long narrow flagellates visible in association with the tailings.

ORF

(cont'd)

Microscopic Examination of Test Cultures

Dec 2/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#3	3.2	High population of T.f. No predators.
#4	6.5	Low population of T.f. Protozoa are large, fast ciliates. Also observed small motile bacterial in vibroid motion.
$8	7.6	Low population of T.f. Ciliates are the only protozoa seen. Large ciliates moved quickly and high number of them.
#5	3	High population of T.f. No protozoa seen.
#6	4.6	High population of T.f. No protozoa seen.
#5	2.2	Moderate decrease in T.f. population. Flagellates seen. They were about twice the width and four times the length of T.f. Tend to spin on one end (uniflagellate) At times flagellum is visible at 400 times magnification.
#16		Low population of T.f. Observed bulb shaped ciliate, slow moving. One ciliate about 20 times length of T.f. Some flagellates 4-5 times longer than T.f. May in fact be bacteria.
#5	2/2.4	High population of T/f. Protozoa may be ciliates. Moved very quickly and were difficult to examine.
#4	6.5	Low T.f. population. Ciliates and bipolar flagellates present but ciliates outnumber flagellates.

ORI

(cont'd)

Microscopic Examination of Test Cultures

Dec 2/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#8	7.5	High T.f. population. A few large ciliates seen.
#14-2	2.5	High T.f. population. Some small fast flagellates.
#3A-2	2.5	High population of T.f. cells. Many small oval shaped protozoa. May be flagellates.

ORF

(cont'd)

Microscopic Examination of Test Cultures

Dec 6/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Subculture of 1	2.6	High T.f. population. No predators observed.
Subculture of 1	3.5	No protozoa seen. High T.f. population.
Original 2	6.0	No protozoa and high T.f. population.
Subculture of 4	6.5	No protozoa observed and T.f. population high.
Subculture of 5-1	2.4	T.f. population high but some flagellates were present in high numbers.
Subculture of 5.2	2.4	Flagellates present in high numbers but T.f. population unchanged.
Subculture of 5-3	2.2	T.f. numbers high but some ciliates and flagellates present.
Subculture of 13	3.1	T.f. population high. Only a few flagellates seen.
Subculture of 14	2.8	T.f. population high. Flagellates and ciliates seen.
Subculture of 14-2	2.5	High T.f. population. Some protozoa seen.
Subculture of 19	3.1	High population of both T.f. and flagellated protozoa.
Subculture of 20	2.9	Again high population of T.f. and flagellated and ciliated protozoa.
Subculture of 11		High T.f. population. Long flagellates seen; slow moving.
Subculture of 3-2	2.5	High T.f. population. Small fast protozoa present.

ORI

(cont'd)

Microscopic Examination of Test Cultures

Dec 6/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#1		High T.f. population. No protozoa.
#2	6.0	High T.f. population. No protozoa.
#3-1	3.2	Only one protozoan seen, but motionless. T.f. population high.
#3-2	3.2	Low numbers of small flagellates and ciliates. T.f. population high.
#4-1	7.0	No predators seen. T.f. population high.
#4-2	7.0	No predators seen. T.f. population high.
#5-1	2	Small circular protozoa. T.f. population high.
#5-2	2	T.f. population high. Oval protozoa (flagellates).
#6	4.6	T.f. population high. No protozoa seen.
#7	1.0	High T.f. population. No protozoa.
#8	7.5	High T.f. population. No protozoa.
#9	7.1	Ciliates present. T.f. population high.
#10	7.1	Many flagellates and ciliates. T.f. population high.
#11	4.2	Observed long thin slow flagellates and fast round ciliates. No change in T.f. population..
#12	9.7	No protozoa. No change in T.f. population.

ORF

(cont'd)

Microscopic Examination of Test Cultures

Dec 6/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
#13	3.3	Pod-shaped predators seen. High T.f. population.
#14	2.7	No protozoa. T.f. population high.
#15	8.3	No protozoa. T.f. population high.
#16	3.1	No protozoa. T.f. population high.
#17	8.4	No protozoa. T.f. population high.
#18	3.2	No protozoa. T.f. population high.
#19	3.3	No protozoa. T.f. population high.
#20	2.7	No protozoa. T.f. population high.
#21		No protozoa. T.f. population high.
Clarkson activated sludge	6.0	High T.f. population. Some flagellates and ciliates (but not as many as expected).

(cont'd)

Microscopic Examination of Test Cultures

Dec 11/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Subculture of 1	2.6	High T.f. population. No protozoa. A lot of debris.
Subculture of 1	3.5	T.f. population lower. Long cells seen (4-5 times length of T.f.). No flagella seen but vibratory motion seen. May not be protozoa.
Subculture of 2	6.0	High population of T.f. Circular flagellates seen displaying slow wide circular movement.
Subculture of 4	6.5	No protozoa seen. T.f. population is high.
Subculture of 5-1	3	T.f. numbers may be decreasing slightly. High population.
Subculture of 5-2	2.4	Looks the same as 5-1
Subculture of 5-3	2.2	Small protozoa present. High population of T.f. cells.
Subculture of 13	3.1	Moderate decrease in T.f. population. No change visually in optical density of culture. Clumps of protozoa almost circular in shape. Look like flagellates.
Subculture of 14	2.8	Clumping of small cells. Thought to be T.f. A few oval flagellates observed.
Subculture of 14-2	2.5	No clumping of cells. T.f. population moderate. Large number of protozoa. rod shaped and circular shapes.
Subculture of 19	3.1	T.f. population was moderate. A high population of circular and oval protozoa. Most were spinning or moving in corkscrew path.

ORF

(cont'd)

Microscopic Examination of Test Cultures

Dec 11/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Subculture of 20	2.9	Oval protozoa with long whip-like flagellum seen. T.f. population moderate.
Subculture of 11		A high population of T.f. cells. No protozoa present.
Subculture of 3A-2	2.5	High T.f. cell numbers. No protozoa seen.

New Table from Dec 11/85
(All from original samples)

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
1		High population of T.f. cells. No protozoa observed.
2	6.0	Observed slow moving pear-shaped flagellated protozoa. Also saw long rod shaped cells. T.f. population was moderate.
3-1	3.2	Protozoa (flagellated) observed. Chains of rod-shaped bacteria also present. Number of T.f. cells high.
3-2	3.2	Same as 3-1 but also some circular protozoa present.
4-1	7.0	A few long rod shaped cells. Not actively motile. T.f. numbers remain high.
4-2	7.0	Observed same type of protozoa as in 4-1 but the population was more motile with higher numbers.
5-1	2.0	A few flagellated protozoa spinning in one spot. T.f. numbers high.
5-2	2.0	Actively motile protozoa but T.f. numbers remain high.

ORF

(cont'd)

Microscopic Examination of Test Cultures

New Table from Dec 11/85
(All from original samples)

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
6	4.6	A high population of mostly circular shaped protozoa. Some clumping of protozoa observed. T.f. numbers are high.
7	1.0	No protozoa. Population of T.f. cells remain high.
8	7.5	Actively motile rod shaped population of protozoa. High T.f. population.
9	7.1	Small circular protozoa spinning in one spot observed. High T.f. numbers still.
10	7.1	Many fast protozoa. T.f. population is low.
11	4.2	No protozoa. High T.f. numbers
12	9.7	High T.f. population. No protozoa.
13	3.3	Oval and circular flagellates. T.f. population moderate.
14	2.7	Many long flagellates. Motion quick. Unflagellated end moves back and forth. T.f. population moderate.
15	8.3	High T.f. numbers. No protozoans.
16	3.1	Small circular flagellates and T.f. numbers were moderate.
17	8.4	Only a few small ciliates seen. No change in T.f. numbers.
18	3.2	Only large fast ciliates seen. T.f. population not decreasing.

(cont'd)

Microscopic Examination of Test Cultures

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
New Table from Dec 11/85 (All from original samples)		
19	3.3	Moderate T.f. numbers. Observed small circular protozoa spinning in one place.
20	2.7	Fast darting oval ciliates. The T.f. numbers were moderate.
21		High T.f. numbers. No protozoa. Debris present in solution.
Clarkson Activated Sludge	6.0	T.f. population was moderate. Many active protozoa in all shapes and sizes.
Dec 13/85		
3A-2	2.5	Several small flagellates per field. No change in high T.f. numbers.
10	5.0	No protozoa. T.f. population high.
10	3.0	T.f. high but clumping. No protozoa.
Clarkson Activated Sludge	5.0 and 3.0	No predators seen. T.f. numbers are moderate.
Dec 17/85		
3-A2	2.5	No protozoa. T.f. still high.
10	5.0	T.f. population decreasing. Flagellates and ciliates visible.
10	3.0	T.f. population high. No protozoa.
Clarkson Activated Sludge	5.0	Low T.f. population. No protozoa but many bacterial clumping.
Clarkson Activated Sludge	3.0	High T.f. population. No active protozoa. Budding yeasts (?) seen.

ORF

(cont'd)

Microscopic Examination of Test Cultures

Dec. 24/85

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Activated Sludge	5.0	Moderate T.f. population. Few protozoa seen. Lots of budding yeasts and hyphae.
Culture 3A-2	3.5	High T.f. population. A lot of protozoa, particularly flagellates.
Oakville Raw Sewage	7.0	High T.f. population. Some cysts.
Jan. 8/86		
Clarkson Activated (from 7/1/86)	7.7	Few protozoa. Some small ciliated. The T.f. population does not appear to be high but the debris present may obscure view.
Clarkson Raw Sewage (from 7/1/86)	7.25	Low T.f. population. Many very large protozoans seen. In particular large rotifers (~20 per loopful). Some flagellates also present. No ciliated forms seen. Rotifers were 400 µm (Philodina).
Clarkson Raw Sewage (from 7/1/86)	5.25	Persistence of rotifers but T.f. population remains high.
Jan. 14/86		
Clarkson Anaerobic Sludge (from 7/1/86)		Some rotifers still present. More ciliates present. Also a few flagellates present but paramecium-type organisms dominate.
Clarkson Raw Sewage (from 7/1/86)	7.25 and 5.25	T.f. population stable. Many long thin flagellates present.
Jan 15/86		
Clarkson Activated Sludge (from 14/1/85)	5.55	Spun down on centrifuge at 2500 rpm for 5 minutes.
Subculture from Clarkson Raw Sewage (7/1/86)	6.5	High T.f. population. Long thread-like organisms. A few ciliates present, also the odd flagellate. (T.f. numbers were low before fresh T.f. cells were added).

ORF

(cont'd)

Microscopic Examination of Test Cultures

Jan 16/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Activated Sludge	5.5	After centrifugation, no rotifers seen. A few ciliates & small flagellates observed.
Subculture of Clarkson Raw Sewage (original 7/1/86)	3.6 and 3.1	High T.f. numbers. A few ciliates but no flagellates or rotifers.
Original Clarkson Raw Sewage (7/1/86)	7.2	A lot of flagellates and ciliates. No rotifers observed may have settled out in sample.
Original Clarkson Raw Sewage (7/1/86)	5.2	Swarming with large ciliates and flagellates, rotifers. Rotifers present. Thiobacillus population was low.
Activated Sludge (14/1/86)	5.55	After 2500 rpm no ciliates, no flagellates, no rotifers, a few thread-like organisms. T.f. still high in numbers.
Jan 17/86		
Clarkson Raw Sewage (17/1/86)	8.20	Centrifuged (2500 rpm) and not centrifuged samples put on.
Clarkson Activated Sludge (17/1/86)	8.20	Centrifuged & not centrifuged samples put on.
Jan 20/86		
Raw Sewage (Clarkson) centrifuged	4.9	Small ciliates but T.f. numbers were still high.
Clarkson Activated Sludge (centrifuged)	3.2	T.f. population high. No protozoa.
Clarkson Activated Sludge (not centrifuged)	3.0	No protozoa. T.f. population high.
Clarkson Raw Sewage (not centrifuged)	4.5	Ciliates and flagellates present. T.f. population high.

(cont'd)

Microscopic Examination of Test Cultures

Jan 21/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Raw Sewage from 15/1/86 (not centrifuged)	5.5	Large protozoa, i.e. vorticella and rotifers. T.f. decreasing.
Clarkson Raw Sewage and Activated Sludge (not centrifuged) (15/1/86)	7.2 & 5.55	No protozoan forms seen. T.f. numbers high.
Fresh Clarkson Raw Sewage (not centrifuged)	7.75	Small protozoans. T.f. population high.
Fresh Clarkson Raw Sewage (centrifuged	5.1 & 5.3	Small ciliates. T.f. population high.
Fresh Clarkson Activated Sludge (not centrifuged)	6.7	Small ciliates. T.f. numbers high.
Fresh Clarkson Activated Sludge (not centrifuged)	4.3	Small ciliates. T.f. numbers high.
Fresh Clarkson Activated Sludge (centrifuged)	3.2 & 4.5	Small ciliates, some narrow long rotifers seen.
Jan 22/86		
Activated Sludge from Clarkson (centrifuged) (21/1/86)	3.2 &	T.f. only seen. No protozoa.
Clarkson Raw Sewage (centrifuged) (21/1/86)	5.1 & 5.3	Some small fast ciliates 20 m long. Also rosette formations of organisms, in tumbling motion.
Clarkson Raw Sewage & Activated Sludge (not centrifuged)	4 to 5	Many sizes of protozoa but large forms have highest numbers. T.f. numbers unchanged.

ORF

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Microscopic Examination of Test Cultures

Jan 23/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Activated Sludge (centrifuged) (21/1/86)	3.2 & 4.5	No protozoa. Only high T.f. population.
Clarkson Raw Sewage (centrifuged) (21/1/86)	4 to 5	20 m flagellates. No change in T.f. population yet. Still clumps of organisms (rosettes). Maybe Gonium pectorale or Synura sp. 20 to 30 organisms per clump.
Jan 24/86		
Clarkson Raw Sewage (centrifuged) (24/1/86)	4.5 & 3.5	Not observed 24/1/86
Clarkson Raw Sewage (not centrifuged) (24/1/86)	5.5	Not observed 24/1/86
Clarkson Activated Sludge (centrifuged) (24/1/86)	6.5 & 6.3	Not observed 24/1/86
Clarkson Activated Sludge (not centrifuged) (24/1/86)	6.5	Not observed 24/1/86
Jan 27/86		
Check on Samples from 21/1/86	Various	No change in rosette formations but no decrease in T.f. population.
Jan 28/86		
Clarkson Raw Sewage (centrifuged) (28/1/86)	3.6 & 4.4	No protozoa. T.f. population high
Clarkson Raw Sewage (not centrefuged) (8/1/86)	3.8	No protozoa. T.f. population high
Clarkson Activated Sludge (centrifuged) (28/1/86)	3.6 & 6.4	No protozoa. T.f. population high
Clarkson Activated Sludge (not centrifuged) (28/1/86)	3.6	No protozoa. T.f. population high.

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Microscopic Examination of Test Cultures

Jan 29.86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Activated Sludge (not centrifuged) 24/1/86	6.5	Many large ciliates, flagellates & rotifers. Low T.f. population.
Clarkson Activated Sludge (centrifuged) 24/1/86	6.5	Many ciliates & rosette forms as well as long thin flagellates.
Clarkson Raw Sewage	.6	No protozoa. High T.f. population.
Jan 30/86 to Feb 6/86		
All current samples		No visual change in optical density.
Feb 7/86		
		Eliminated old inactive protozoa/T.f. cultures.
Clarkson Raw Sewage and Activated Sludge		Set up fresh samples with T.f. cells.
Feb. 10/86		
Clarkson Raw Sewage (centrifuged) 7/2/86		Some small flagellates & ciliates present. T.f. numbers seem stable. Also observed a few large ciliates. Perhaps centrifugation was not sufficient.
Clarkson Raw Sewage (not centrifuged) 7/2/86		Long thin flagellates and huge protozoa (ciliated), some small ciliates. No worms seen. T.f. population remains high.
Clarkson Activated Sludge (not centrifuged) 7/2/86		Large sludge worms. Few small ciliates and flagellates.
Clarkson Activated Sludge (centrifuged) 7/2/86		Small flagellates. T.f. seem to be decreasing. More T.f. added to sustain protozoa.

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Microscopic Examination of Test Cultures

Feb 1/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS	
Fresh Samples	Original pH	Low pH	High pH
Clarkson Raw Sewage (centrifuged)	7.4	4.90	6.80
Clarkson Activated Sludge (centrifuged)	7.1	4.05	6.90
Oakville Raw Sewage (centrifuged)	7.5	3.95	6.85
Clarkson Raw Sewage (1 hr. settled)	7.40	5.30	6.90
Clarkson Activated Sludge (1 hr. settled)	7.10	4.05	6.75
Oakville Raw Sewage (1 hr. settled)	7.55	4.70	6.85
Clarkson Raw Sewage (mixed)	7.40	5.45	6.95
Clarkson Activated Sludge (mixed)	7.15	6.20	6.55
Oakville Raw Sewage (mixed)	7.50	5.60	7.00

Feb 12-14/86

Examined all current samples	No sign of visual change in optical density. Raw sewage Clarkson & Oakville and Clarkson Activated Sludge that were centrifuged showed no protozoan activity.

Feb 17/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Raw Sewage 11/2/86	4.9	Mixed culture. Slight decrease in optical density. Large & small ciliates & flagellates. Needs to be recentrifuged.
Clarkson Activated Sludge 11/2/86		Only one small ciliate seen.
Oakville Raw Sewage 11/2/86	3.95	Some clearing. Mixed culture. Large ciliates present. Needs to be centrifuged again.
Post centrifuging Clarkson Raw sewage 11/2/86	4.9 to 3.95	Centrifuged at 5000 rpm for 10 mins. No large ciliates seen. Added more T.f.

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Microscopic Examination of Test Cultures

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Oakville Raw Sewage 11/2/86	3.95	Still some large ciliates. Centrifuged again at 5000 rpm.
Clarkson Raw Sewage (settled) 11/2/86	5.3	Very active mixed culture. Large ciliates dominated. OD reduced.
Clarkson Activated Sludge (settled) 11/2/86	3.95	Active culture. All small ciliates & flagellates but OD not reduced to any extent.
Oakville Raw Sewage (settled) 11/2/86	4.7	Very active mixed culture. Optical density reduced but predominantly large ciliates. Needs centrifuging.
Clarkson Activated Sludge (centrifuged) 28/1/86		No protozoa
Clarkson Raw Sewage (centrifuged) 28/1/86		No protozoa
Oakville Raw Sewage (centrifuged) 11/2/86	Final pH 3.5	Centrifuged at 5000 rpm. Small ciliates & flagellates present. Fresh T.f. cells added.
Oakville Raw Sewage (settled) 11/2/86	Final pH 4.4	Centrifuged at 5000 rpm. Small ciliates & flagellates. A few larger ciliates still present. Added fresh T.f. cells.
Feb 19/86		
Oakville Raw Sewage Centrifuged 11/2/86 Recentrifuged 17/2/86	3.55	Mixture of small ciliates & flagellates. All large ciliates gone.
Clarkson Raw Sewage Centrifuged 11/2/86 Recentrifuged 17/2/86	3.5	Mixed culture of small flagellates & ciliates
Oakville Raw Sewage Settled 11/2/86 Centrifuged 17/2/86		Mixed culture, mostly small flagellates. No large species. Same as Oakville centrifuged Raw Sewage.
Clarkson Activated Sludge 11/2/86 Centrifuged	5.3	Active culture now. Mixed ciliates & flagellates all relatively small. Optical density still high.

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Microscopic Examination of Test Cultures

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Raw Sewage (settled) 11/2/86		Very active mixed culture. High population of larger ciliates. Also high population of flagellates. Should be centrifuged.
Oakville Raw Sewage (settled) 11/2/86	4.7	Active mixed culture. Large ciliates Needs to be centrifuged.
Clarkson Activated Sludge (settled) 11/2/86		Very active flagellate culture.
Oakville Raw Sewage (mixed) 11/2/86		Mixed culture but not as good as others.
Clarkson Raw Sewage (settled) 11/2/86		Relatively clear. Black ppte formed. Active culture of predominantly larger ciliates but flagellates also present in good numbers.
Feb 20/86		
Oakville Raw Sewage Settled 11/2/86 Centrifuged 17/2/86		Almost clear today but larger ciliates re-established. Centrifuged again at 5000 rpm for 10 minutes. Added fresh T.f. cell suspension.
Feb 27/86		
Clarkson Raw Sewage Centrifuged 11/2/86	3.5	Clumping of cells in rosettes. Flagellated protozoans. T.f. population still high.
Clarkson Raw Sewage Centrifuged 11/2; 17/2	5.5	Small flagellates. T.f. population dropping.
Oakville Raw Sewage Centrifuged 11/2; 17/2; 20/2	3.3	Large ciliates re-established. Some small flagellated whiplike motion. T.f. population high.
Oakville Raw Sewage Settled 11/2 Centrifuged 17/2		No protozoa. T.f. population high.

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Microscopic Examination of Test Cultures

March 4/ 86 Observed March 5/86

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Fresh Clarkson Raw Sewage, settled	7.10	Long thin stick-like flagellates. Very active small flagellates, too. High T.f. population.
Clarkson Activated Sludge, settled	6.90	Thin flagellates. High T.f. population.
Oakville Raw Sewage, settled	7.05	Low protozoan population. T.f. numbers still high.
Clarkson Raw Sewage, mixed	7.05	Large flagellates only.
Clarkson Activated Sludge, mixed	6.85	Will centrifuge, rotifers present.
Oakville Raw Sewage, mixed	7.10	Only large flagellates.
Subculture of Clarkson Raw Sewage, Centrifuged 11/2; 17/2	7.00	Active flagellates present.
Clarkson Raw Sewage 11/2; centrifuged 17/2	6.90	Fresh T.f. cells added. Active flagellates present.
March 6/86		
Clarkson Raw Sewage settled 4/3/86	7.10	Two large flagellates seen. All others small with phase bright internal ultrastructures. T.f. population not changed.
Clarkson Activated Sludge, settled 4/3/86	6.90	Still only small flagellates seen but no sign of predation.
Oakville Raw Sewage settled 4/3/86	7.05	Larger ciliates now appearing. No change in T.f. population.
Mixed Clarkson Raw Sewage	7.05	Larger organisms; should be centrifuged. No change in T.f. numbers.
Mixed Clarkson Activated Sludge	6.85	
Mixed Oakville Raw Sewage	7.10	

ORF

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Microscopic Examination of Test Cultures

SAMPLE IDENTIFICATION	ORIGINAL pH	OBSERVATIONS
Clarkson Raw Sewage 11/2; centrifuged 17/2 Subcultured and Clarkson Raw Sewage 11/2; centrifuged 17/2	7.00	Black precipitate in bottom of tubes. T.f. numbers high. Predator numbers unchanged.
March 7, 10 and 13/86		
Examined all cultures		Black precipitate forming in all tubes. Dropped pH of Clarkson Raw Sewage (centrifuged 11/2; 17/2) to pH 5.0.
March 18 and 19/86 Observations 21/3/86		
Fresh samples		
Clarkson Raw Sewage, centrifuged	7.50	No protozoa except large ciliates.
Clarkson Activated Sludge, centrifuged	7.35	Large flagellates. Good predation.
Oakville Raw Sewage, centrifuged	7.20	Black ppte. Many protozoa.
Starch Processing Plant Effluent, centrifuged	7.00	Squiggly flagellates, some ciliates. High bacterial population (rods & cocci).
Clarkson Raw Sewage, mixed	7.50	
Clarkson Activated Sludge, mixed	7.35	Some large rotifers. Lots of flagellates & ciliates
Oakville Raw Sewage, mixed	7.20	
Starch Processing Plant Effluent, mixed	7.00	Round ciliates & flagellates. No change in T.f. population
Chicken Processing Plant, centrifuged	7.20	Lots of fat and garbage. Relatively large flagellates, slow moving. No decrease in T.f. numbers. Black ppte in tubes.
Chicken Processing Plant, mixed	7.00	

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APPENDIX III

FIGURES

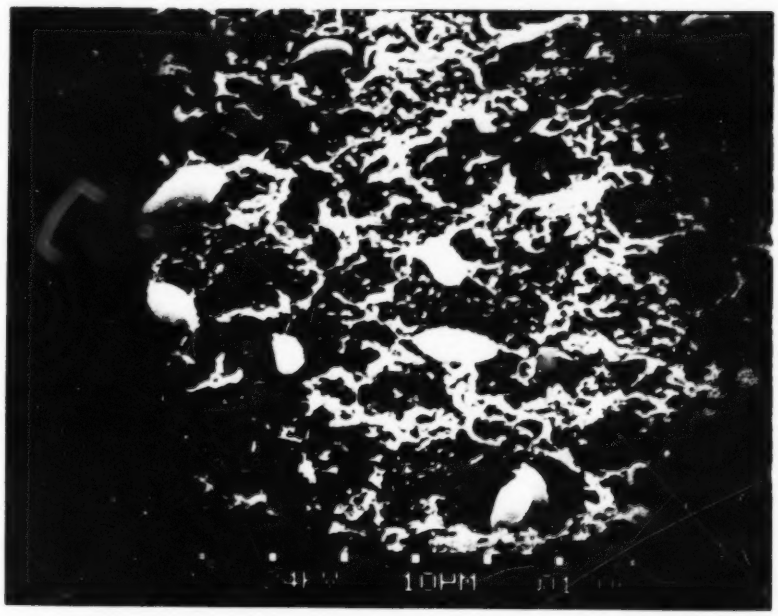

FIG. 1: Mixed culture of protozoa in T. ferrooxidans suspension pH 7.2, inoculated with centrifuged sample of primary sludge from chicken processing plant (Scanning Electron Micrograph)

FIG. 2: Mixed culture of protozoa in T. ferrooxidans suspension pH 7.2, inoculated with centrifuged sample of primary sludge from chicken processing plant (Scanning Electron Micrograph)

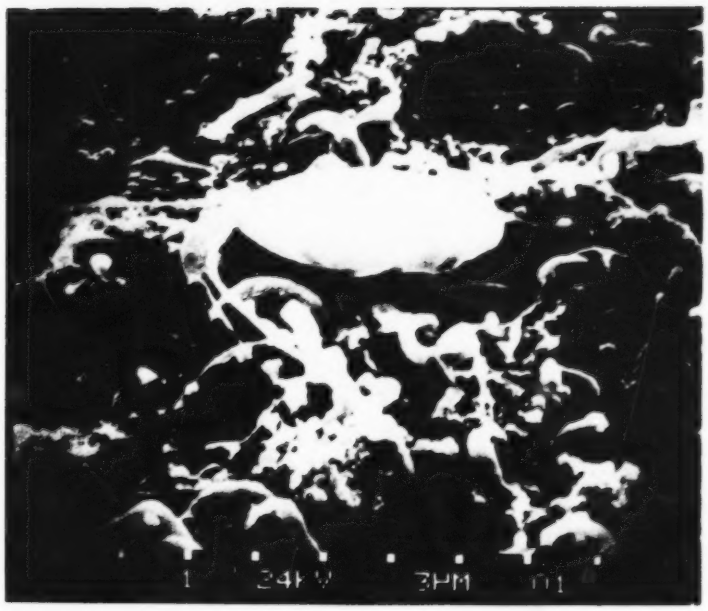

FIG. 3: Flagellated protozoan in T. ferrooxidans suspension pH 7.2, inoculated with centrifuged primary sludge from chicken processing plant (Scanning Electron Micrograph)

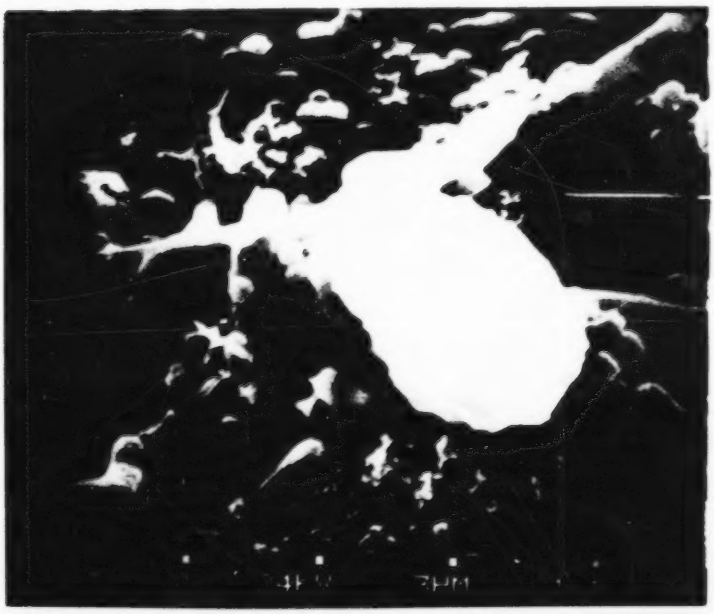

FIG. 4: A second flagellated protozoan observed in a T. ferrooxidans suspension pH 7.2, inoculated with centrifuged primary sludge from chicken processing plant (Scanning Electron Micrograph)

APPENDIX IV

BIBLIOGRAPHY

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McCready, R.G.L. (1984). Personal communication